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Symbiotic Hydrogenase Activity in *Bradyrhizobium* sp. (*Vigna*) Increases Nitrogen Content in *Vigna unguiculata* Plants

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***Bradyrhizobium* sp. (*Lupinus*) and *Bradyrhizobium* sp. (*Vigna*) mutants in which hydrogenase (*hup*) activity was affected were constructed and analyzed. *Vigna unguiculata* plants inoculated with the *Bradyrhizobium* sp. (*Vigna*) *hup* mutant showed reduced nitrogenase activity and also a significant decrease in nitrogen content, suggesting a relevant contribution of hydrogenase activity to plant yield.**

Bacteria belonging to the group collectively known as rhizobia form nitrogen-fixing nodules in symbiosis with leguminous plants. In these symbioses, a large amount of hydrogen is released from nodules as a by-product of the nitrogenase reaction. Certain rhizobial strains are able to oxidize this hydrogen through the expression of uptake hydrogenases. Hydrogen recycling has been shown to reduce energy losses associated with diazotrophy (19). Consequently, incorporation of the hydrogen oxidation capability into rhizobial strains has been proposed as a way to improve symbiotic nitrogen fixation (11). However, enhancement of legume host productivity associated to the activity of the hydrogenase enzyme has been reported only for certain symbiotic associations, such as *Bradyrhizobium japonicum*/soybean (1, 8).

To date, a detailed molecular characterization of hydrogenase gene clusters has been reported for *Rhizobium leguminosarum* bv. viciae, *B. japonicum*, and *Azorhizobium caulinodans* (3, 11, 16). Hydrogenase gene clusters share a common core of 18 genes, named *hup*, *hyp*, and *hox* genes, that encode functions involved in enzyme biosynthesis and regulation. In certain rhizobia, hydrogenase activity is induced in microaerobic free-living cells, in addition to symbiotic conditions (17, 21). In previous work, we analyzed the presence of *hup*, *hyp*, and *hox* genes in several *Bradyrhizobium* sp. (*Lupinus*) and *Bradyrhizobium* sp. (*Vigna*) strains (2). Significant differences in the presence of *hup* regulatory genes were observed by Southern hybridization assays. In addition, phylogenetic analysis of partial *hupS* and *hupL* sequences revealed that *Bradyrhizobium* sp. (*Vigna*) *hup* sequences cluster apart from those of *Bradyrhizobium* sp. (*Lupinus*). In the light of these differences within the *Bradyrhizobium* genus, we have characterized the symbiotic hydrogenase activity of the previously studied *Bradyrhizobium* sp. (*Lupinus*) and *Bradyrhizobium* sp. (*Vigna*) strains and the relevance of their hydrogenase systems for plant productivity.

The hydrogen oxidation capabilities of several *Bradyrhizobium* sp. (*Lupinus*) and *Bradyrhizobium* sp. (*Vigna*) strains were analyzed in symbiosis with *Lupinus albus* cv. Unicrop and *Vigna unguiculata* cv. Blackeye, respectively. Plants inoculated with bacterial cultures were grown under bacteriologically controlled conditions as previously described (9), by using a nitrogen-free plant nutrient solution supplemented with 20 μM $NiCl_2$ in order to optimize hydrogenase activity (6). Hydrogen metabolism was characterized by measuring hydrogen evolution in intact nodules (5) and hydrogenase activity in bacteroid suspensions (15). In this analysis, we found that the *Bradyrhizobium* sp. (*Lupinus*) strains displayed a wide range of hydrogen uptake activity levels (Table 1). Strains 624 and Z89 showed the lowest and highest levels of hydrogenase activity, respectively, whereas strains UPM860, 466, and IM43B exhibited intermediate values. None of them was able to fully recycle the hydrogen produced by nitrogenase, since nodules evolved hydrogen in all cases (Table 1). Since a significant plant host effect on hydrogenase activity has been previously shown (12), assays were also carried out using *Ornithopus compressus* as a plant host. However, similar values of bacteroid hydrogenase activity were obtained in this legume host (data not shown). For *Bradyrhizobium* sp. (*Vigna*) strains, different levels of symbiotic hydrogenase activity were also observed, but in this case, a large proportion of strains recycled all the hydrogen generated in the nitrogen fixation process (Table 1). Hydrogenase activities from *Bradyrhizobium* sp. (*Lupinus*) and *Bradyrhizobium* sp. (*Vigna*) strains were similar to those previously obtained without nickel supplementation (12, 14). Due to the higher levels of hydrogenase activity exhibited by *Bradyrhizobium* sp. (*Lupinus*) Z89 and *Bradyrhizobium* sp. (*Vigna*) M5, we selected these two strains for further functional characterization of the hydrogen uptake system.

To generate the *Bradyrhizobium* sp. (*Lupinus*) Z89.1 and *Bradyrhizobium* sp. (*Vigna*) M5.1 *hupSL* mutant strains, 1.5-kb DNA fragments containing both the *hupS* 3′ end and the *hupL* 5′ end from the Z89 and M5 wild-type strains were obtained by PCR amplification by using the degenerate primers hupSL1

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TABLE 1. Hydrogen metabolism in *Bradyrhizobium* Hup^+ strains

Strain/host	Bacteroid hydrogenase activity[a]	Nodule H_2 evolution[b]
Bradyrhizobium sp. (*Lupinus*)/*L. albus*		
UPM860	510 ± 30	4.1 ± 0.6
624	50 ± 10	5.3 ± 0.8
466	820 ± 60	0.5 ± 0.2
Z89	2,070 ± 160	0.3 ± 0.1
IM43B	880 ± 10	0.6 ± 0.4
Bradyrhizobium sp. (*Vigna*)/*V. unguiculata*		
M2	350 ± 20	0.4 ± 0.1
M5	1,500 ± 240	<0.1
M18	1,120 ± 170	<0.1
M21	1,080 ± 210	<0.1
M43	840 ± 10	<0.1
B78	1,480 ± 50	<0.1
B96	520 ± 30	0.2 ± 0.1
B97	20 ± 10	3.6 ± 0.1
UPM938	510 ± 110	<0.1

[a] Hydrogenase activities were determined in bacteroid suspensions and are expressed in nmol H_2 h^{-1} (mg prot)$^{-1}$. Protein content of bacteroid suspensions was determined by the bicinchoninic acid method (20). Values are the averages of three independent determinations ± standard errors.

[b] Values (μmol H_2 h^{-1} g^{-1} fresh weight of nodules) are the averages of three independent determinations with two replicates ± standard errors. Values of <0.1 indicate rates of hydrogen evolution not detectable with the amperometric method.

and hupSL2 and PCR conditions previously described (2). Each PCR product was cloned in the PCR2.1-TOPO vector (Invitrogen BV, Groningen, The Netherlands), excised with KpnI-XbaI, and introduced into the pSS10 vector (3). The resulting plasmids pSCB2 and pSCB3 were introduced by conjugation into strains Z89 and M5, respectively, and transconjugants were selected in *Rhizobium* minimal medium (13) plates supplemented with spectinomycin. Genomic DNA of transconjugant strains was analyzed by Southern hybridization experiments (18) using a *Rhizobium leguminosarum hupS* gene probe in order to confirm the single-crossover event leading to the plasmid integration in the corresponding *hupSL* region (data not shown).

The effect of *hupSL* gene disruption in *Bradyrhizobium* sp. (*Lupinus*) Z89.1 and *Bradyrhizobium* sp. (*Vigna*) M5.1 mutants was assessed by measuring hydrogenase activity in symbiosis with *L. albus* and *V. unguiculata* plants, respectively (Table 2). Bacteroids from both mutant strains exhibited basal levels of hydrogenase activity compared with those of the corresponding wild-type strains. In agreement with this, nodules from *L. albus* and *V. unguiculata* induced by these mutants evolved large amounts of hydrogen. These results confirmed inactivation of the hydrogenase system and the absence of other functional *hup* systems in these strains.

TABLE 3. Nitrogen content and dry weight of *Vigna unguiculata* plants inoculated with the *Bradyrhizobium* sp. (*Vigna*) wild-type M5 and *hupSL* mutant M5.1 strains in three independent experiments[a]

Experiment	Strain	30 days		40 days	
		N content	Dry weight	N content	Dry weight
I	M5	58.4a	1.31a	81.4a	2.48a
	M5.1	40.7b	1.24a	74.3b	2.46a
	Uninoculated	6.11c	0.48b	2.91c	0.56b
II	M5	46.7a	1.25a	113.1a	3.64a
	M5.1	30.2b	0.94b	102.4b	4.71a
	Uninoculated	6.05c	0.43c	2.53c	0.61b
III	M5	ND	ND	81.1a	3.31a
	M5.1	ND	ND	70.2b	3.02a
	Uninoculated	ND	ND	2.35c	0.49b

[a] Determinations were carried out after 30 or 40 days of cultivation. Nitrogen content (determined with a Kjeldhal apparatus) is expressed as mg plant^{-1} and dry weight (measured after drying for 48 h at 80°C) in g plant^{-1}. Values are averages of eight determinations. Different letters within the same column and experiment indicate significant differences at 1% based on Duncan's test. ND, not determined.

Disruption of *hupSL* genes induced a differential effect on the levels of nitrogenase activity of the *Bradyrhizobium* sp. (*Vigna*) M5.1 and *Bradyrhizobium* sp. (*Lupinus*) Z89.1 *hup* mutants (Table 2). Acetylene reduction activity in *Bradyrhizobium* sp. (*Vigna*) M5.1 was significantly reduced compared with that of the wild-type strain, whereas no difference between values from *Bradyrhizobium* sp. (*Lupinus*) Z89 and mutant strain Z89.1 was observed. These results indicate that hydrogenase activity might be necessary for optimal levels of nitrogenase activity in *Bradyrhizobium* sp. (*Vigna*) bacteroids.

To test whether the observed reduction of nitrogenase activity in *Bradyrhizobium* sp. (*Vigna*) M5.1 bacteroids could affect legume productivity, we analyzed the effect of hydrogenase inactivation on nitrogen accumulation and dry weight of the corresponding plant host. *V. unguiculata* plants inoculated with the *Bradyrhizobium* sp. (*Vigna*) M5.1 mutant showed levels of nitrogen accumulation lower than those inoculated with the wild-type strain (Table 3). This result was consistently observed in five independent experiments carried out with 30- and 40-

TABLE 2. Symbiotic nitrogen fixation and H_2 metabolism of *hupSL* mutants from *Bradyrhizobium* sp. (*Lupinus*) Z89 and *Bradyrhizobium* sp. (*Vigna*) M5[a]

Strain	Genotype	Hydrogenase activity[b]	H_2 evolution[c]	Nitrogenase activity[d]	RE[e]
Z89	Wild type	1,740 ± 260	0.3 ± 0.2	21.3 ± 0.2	0.99
Z89.1	Z89 *hupSL*::*pSCB2*	50 ± 10	5.3 ± 0.4	21.5 ± 2.8	0.76
M5	Wild type	1,710 ± 70	<0.1	20.3 ± 2.6	1
M5.1	M5 *hupSL*::*pSCB3*	40 ± 10	3.9 ± 0.6	15.8 ± 1.2	0.75

[a] Values were determined 30 days after inoculation.

[b] Hydrogenase activities were determined in bacteroid suspensions and are expressed in nmol H_2 h^{-1} (mg prot)$^{-1}$. Values are averages of four determinations with two replicates ± standard errors.

[c] Expressed as μmol H_2 h^{-1} g^{-1} (fresh weight of nodules). Values are averages of four determinations with two replicates ± standard errors.

[d] Expressed as μmol C_2H_2 reduced h^{-1} g^{-1} (fresh weight of nodules). Values are averages of four determinations with four replicates ± standard error.

[e] Relative efficiency (RE) was calculated as 1 − (H_2 evolution/C_2H_2 reduction).

TABLE 4. Nitrogen content and dry weight of *Lupinus albus* plants inoculated with *Bradyrhizobium* sp. (*Lupinus*) wild-type and *hupSL* mutant strains[a]

Strain	Experiment I		Experiment II	
	N content	Dry weight	N content	Dry weight
Z89	43.5a	1.03a	90.2a	2.24a
Z89.1	43.7a	1.03a	90.3a	2.16a
Uninoculated	8.5b	0.52b	7.7b	0.67b

[a] Determinations were carried out after 40 days of cultivation. Nitrogen content is expressed as mg plant^{-1} and dry weight in g plant^{-1}. Values are averages of eight determinations. Different letters in the same column indicate significant differences at 1% based on Duncan's test.

day-old plants grown under controlled chamber conditions. The reduction in nitrogen content was also correlated with a reduction of plant dry weight in one experiment at 30 days (Table 3). Similar experiments were carried out in the *Bradyrhizobium* sp. (*Lupinus*)-*L. albus* symbiosis. In this case, no significant difference in nitrogen content or dry weight was observed between 40-day-old *L. albus* plants inoculated with the *Bradyrhizobium* sp. (*Lupinus*) Z89.1 *hup* mutant or the wild-type strain Z89 (Table 4).

Our results show that inactivation of hydrogenase genes impairs bacterial nitrogenase activity in *Bradyrhizobium* sp. (*Vigna*) and has an effect on total nitrogen content of the symbiotic plant partner, *V. unguiculata*. Several mechanisms by which hydrogenase activity might increase the efficiency of the nitrogen fixation process have been proposed (7). Among them, prevention of H_2 inhibition of the nitrogenase reaction, protection of nitrogenase from the O_2 damage, and provision of an additional source of energy appear as critical factors that might influence nitrogenase activity. At this point of the investigation, it is difficult to determine whether one of these factors or a combination of them is responsible for the beneficial effect of hydrogenase activity on the nitrogen fixation process in this specific symbiosis. Such analysis requires further knowledge of the system, and we are now pursuing the molecular characterization of the *Bradyrhizobium* sp. (Vigna) *hup* cluster. These data will hopefully shed some light on the role of hydrogen recycling in this symbiosis, and they also might provide some clues to explain why a very high proportion of *Bradyrhizobium* sp. (*Vigna*) isolates from *Vigna unguiculata* nodules are hydrogenase positive (4, 14).

The use of mutant strains carrying antibiotic resistance genes is somewhat controversial (10), and it can be argued that the resistance markers might unbalance bacterial fitness and affect the nitrogen fixation process. In this work, however, the *Bradyrhizobium* sp. (*Lupinus*) Z89.1 *hup* mutant carries the same resistance gene as *Bradyrhizobium* sp. (*Vigna*) M5.1, and no significant differences either in acetylene reduction assays or in the nitrogen content of *Lupinus* plants were associated with the *hup* mutant in the same experimental conditions. Similarly, no effect on plant productivity was detected in the *A. caulinodans-Sesbania rostrata* symbiosis using a *hup* mutant strain carrying this antibiotic resistance cassette (3). Future work will be focused on the construction and testing of a *hup* deletion mutant in plant productivity experiments to confirm these data and definitely establish the contribution of the hydrogenase system to the productivity of the *Bradyrhizobium* sp. (*Vigna*)-*V. unguiculata* association.

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